

6

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
2 August 2001 (02.08.2001)

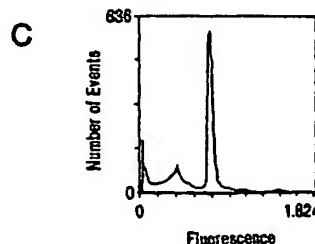
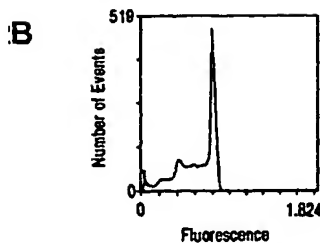
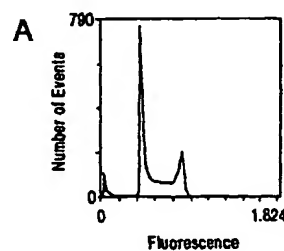
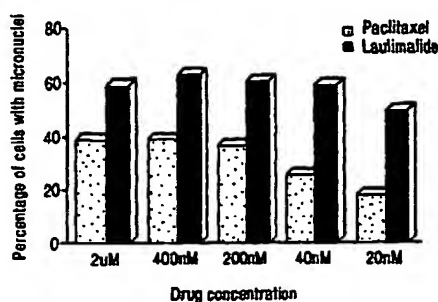
PCT

(10) International Publication Number
WO 01/54689 A1

- (51) International Patent Classification⁷: A61K 31/365, A61P 35/00
- (21) International Application Number: PCT/US01/02590
- (22) International Filing Date: 26 January 2001 (26.01.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
09/493,897 28 January 2000 (28.01.2000) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/493,897 (CON)
Filed on 28 January 2000 (28.01.2000)
- (71) Applicants (for all designated States except US): UNIVERSITY OF HAWAII [US/US]; 2800 Woodland Drive, Suite 280, Honolulu, HI 96822 (US). UTAH STATE UNIVERSITY [US/US]; 1770 North Research Park Way, Logan, UT 84341 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): MOOBERRY, Susan, L. [US/US]; 15742 Dawn Crest, San Antonio, TX 78248 (US). DAVIDSON, Bradley, S. [US/US]; 901 Orchard Drive, River Heights, UT 84321 (US).
- (74) Agents: REITER, Stephen, E. et al.; Foley & Lardner, 23rd Floor, 402 W. Broadway, San Diego, CA 92101-3542 (US).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,

[Continued on next page]

(54) Title: LAULIMALIDE COMPOUNDS AS MICROTUBULE STABILIZING AGENTS



(57) Abstract: Methods of inhibiting the proliferation of hyperproliferative mammalian cells having a multiple drug resistant phenotype utilizing an amount of a laulimalide compound effective to disrupt the dynamic state of microtubule polymerization and depolymerization to arrest cell mitosis are disclosed, together with laulimalide compounds (and compositions containing same) which find use in such methods.

WO 01/54689 A1

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

LAULIMALIDE COMPOUNDS AS MICROTUBULE STABILIZING AGENTS

FIELD OF THE INVENTION

The present invention relates to methods for the treatment of hyperproliferative cells, especially cells having a multiple drug resistance
5 phenotype. In a particular aspect, the present invention relates to methods for the treatment of cancer. In another aspect, the present invention relates to compounds and compositions useful for the above-described methods of treatment.

BACKGROUND OF THE INVENTION

10 Neoplastic diseases or cancers, characterized by the proliferation of cells not subject to normal growth regulation, are a major cause of death in humans. An estimated 1,221,800 new cases and 561,000 deaths were expected to occur in 1999. Lung cancer remains the leading cause of cancer-related deaths in the United States; the estimated 158,900 deaths would
15 account for 28% of the total.

Clinical experience in chemotherapy has demonstrated that new and more effective cytotoxic drugs are desirable to treat these diseases. Such experience has also demonstrated that drugs which disrupt the microtubule
20 system of the cytoskeleton can be effective in inhibiting the proliferation of neoplastic cells.

The microtubule system of eucaryotic cells is a major component of the cytoskeleton and is in a dynamic state of assembly and disassembly; that
25 is, heterodimers of tubulin are polymerized to form microtubules, and microtubules are depolymerized to their constituent components. Microtubules play a key role in the regulation of cell architecture, metabolism, and division, and the dynamic state of the microtubules is critical to their

- 2 -

normal function. With respect to cell division, tubulin is polymerized into microtubules that form the mitotic spindle. The microtubules are then depolymerized when the role of the mitotic spindle has been fulfilled. Accordingly, agents which disrupt the polymerization or depolymerization of microtubules, and thereby inhibit cell growth, comprise some of the most effective chemotherapeutic agents in clinical use.

Such anti-mitotic agents or poisons all kinetically inhibit the normal dynamics of microtubules. There are subtle differences between certain classes of anti-microtubule agents based on their molecular mechanism of action. Colchicine, for example, binds to soluble tubulin and then is incorporated into a growing microtubule, while vinblastine binds to the microtubule end and thereby suppresses microtubule dynamics. At high concentrations, both colchicine and vinblastine cause the loss of cellular microtubules. Paclitaxel (more commonly known as Taxol™) and related taxanes also inhibit microtubule dynamics, yet at high concentrations these agents cause an increase in polymerized tubulin in the cell and thick microtubule bundles are formed.

Paclitaxel was first isolated in 1971 from the bark of the Pacific yew tree (*Taxus brevifolia*), and was approved in 1992 by the US Food and Drug Administration for treatment of metastatic ovarian cancer and later for breast cancer. Paclitaxel has attracted unusually strong scientific attention, not only because of its unique anti-proliferative mechanism of action, but also because it is active against a broad range of tumors. The discovery of the effectiveness of the natural product paclitaxel has led to the production and testing of semisynthetic congeners including docitaxel (Taxotere). These compounds, taxanes, are now recognized as a new class of anti-cancer compounds.

- 3 -

One drawback of paclitaxel is its extreme insolubility: Paclitaxel can be administered effectively only in a solvent including cremophor, which combination can provoke severe hypersensitive immune responses. As a
5 result of these drawbacks, it is considered desirable to investigate the potential utility of other naturally-occurring compounds with similar modes of action as possible anti-cancer compounds.

In addition, merely having activity as an antimitotic agent does not
10 guarantee efficacy against a tumor cell, and certainly not a tumor cell which exhibits a drug-resistant phenotype. For example, vinca alkaloids, such as vinblastine and vincristine, and taxanes are effective against neoplastic cells and tumors, yet they lack or display reduced activity against drug-resistant tumors and cells. One basis for neoplastic cells to display drug resistance
15 (DR) or multiple-drug resistance (MDR) is through the over-expression of P-glycoprotein. Compounds which are poor substrates for transport of P-glycoprotein should be useful to circumvent such DR or MDR phenotypes.

Accordingly, expression of the DR or MDR phenotype by many tumor
20 cells and the clinically proven mode of action of anti-microtubule agents against neoplastic cells necessitates the development of anti-microtubule agents which are cytotoxic to non-drug resistant neoplastic cells as well as being cytotoxic to neoplastic cells with a drug resistant phenotype.

25 Since the discovery of the mechanism of action of paclitaxel, only three other non-taxane chemical classes have been identified that possess a similar mode of action (i.e., epothilones A and B, discodermolide, and eleutherobin and related sarcodictyins A and B). The epothilones were isolated from the myxobacterium *Sorangium cellulosum* as a result of a large-
30 scale screening effort. The epothilones have generated significant interest, as they retain activity against drug-resistant cell lines. Epothilones have been

- 4 -

isolated from a species of bacteria found in soil samples collected from the banks of the Zambesi River in the Republic of South Africa, and have been recently synthesized.

5 Discodermolide was purified from the marine sponge *Discodermia dissoluta* as an immunosuppressant and was screened for anti-mitotic activity on the basis of a predictive structure-activity relationship when compared with other tubulin-interacting drugs. Discodermolide promotes tubulin assembly more potently than paclitaxel and it is an effective inhibitor of cell
10 growth in paclitaxel-resistant cells.

 Eleutherobin, a potent cytotoxin from the soft coral *eleutherobia* sp., promotes tubulin polymerization but exhibits cross-resistance to paclitaxel-resistant cell lines. The potential therapeutic usefulness of these new
15 microtubule-stabilizing compounds, and whether they will provide advantages over the taxanes, have yet to be determined.

 Accordingly, it remains a goal in the art to identify additional naturally-occurring compounds with modes of action similar to the taxanes, but which
20 display different tissue specificity, solubility, and/or activity against drug-resistant, and particularly multiple-drug resistant, tumors and cells.

Brief Description of the Invention

 In accordance with the present invention, it has been discovered that
25 laulimalides and related compounds are potent inhibitors of cell proliferation. Accordingly, there are provided methods of inhibiting the proliferation of hyperproliferative mammalian cells, especially cells having a multiple drug resistant phenotype. Invention methods comprise contacting the cell with an amount of a laulimalide compound effective to disrupt the dynamic state of
30 microtubule polymerization and depolymerization to arrest cell mitosis, thereby inhibiting the proliferation of the cell.

In addition, novel compounds and compositions containing same are also provided. These and other aspects of the present invention will be readily apparent from the following more detailed description.

5

Brief Description of the Drawings

The invention will be better understood by reference to the following detailed description of specific embodiments when considered in combination
10 with the drawings that form a part of this specification.

Figure 1 is a graphic representation comparing the effects of Laulimalide and paclitaxel on micronuclei formation, in which A-10 cells were treated for 18 hours with Laulimalide or paclitaxel, the cells were fixed and
15 nuclei were visualized by DAPI staining.

Figure 2 collectively is a graphic representation comparing the effects of Laulimalide on cell cycle distribution, wherein log phase growth cultures of MDA-MB-435 cells were treated, the cells were fixed, stained, and analyzed
20 on a Coulter EPICS XL-MCL flow cytometer and plotted as events versus propidium iodide fluorescence intensity.

Figure 2A portrays control cells.

25 Figure 2B portrays the cells treated with 20nm Laulimalide for 9 hours.

Figure 2C portrays the cells treated with 20nm Laulimalide for 18 hours.

- 6 -

Figure 3 collectively is a graphic representation comparing the effects of Laulimalide and paclitaxel on tubulin polymerization, in which the polymerization of bovine brain tubulin was monitored.

5

Figure 3A portrays the cells treated with Laulimalide.

Figure 3B portrays the cells treated with paclitaxel.

10

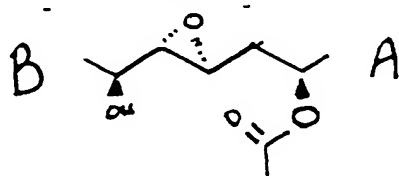
Detailed Description of the Invention

In accordance with the present invention, there are provided methods of inhibiting the proliferation of hyperproliferative mammalian cells having a multiple drug resistant phenotype. Invention methods comprise contacting such cells with an amount of a laulimalide compound effective to disrupt the dynamic state of microtubule polymerization and depolymerization to arrest cell mitosis, thereby inhibiting the proliferation of said cells.

15

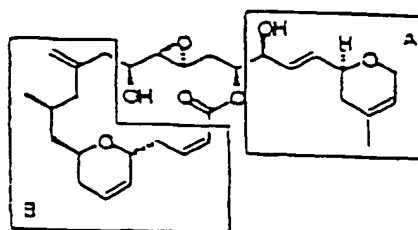
Laulimalide compounds contemplated for use in the practice of the present invention can be described by Structure I as follows:

20



in which A and B are structural variants of Laulimalide regions A and B as follows:

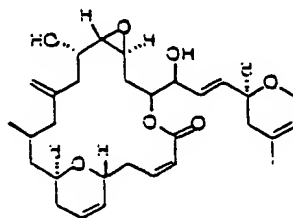
25



- 7 -

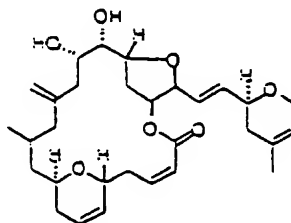
wherein the epoxide ring in Structure I can optionally be replaced with a double bond. Such variants preserve the ability to inhibit the proliferation of a hyperproliferative mammalian cell having a multiple drug resistant phenotype.

5 Specific embodiments of compounds which are contemplated for use in the practice of the present invention are represented here by laulimalide:



Laulimalide

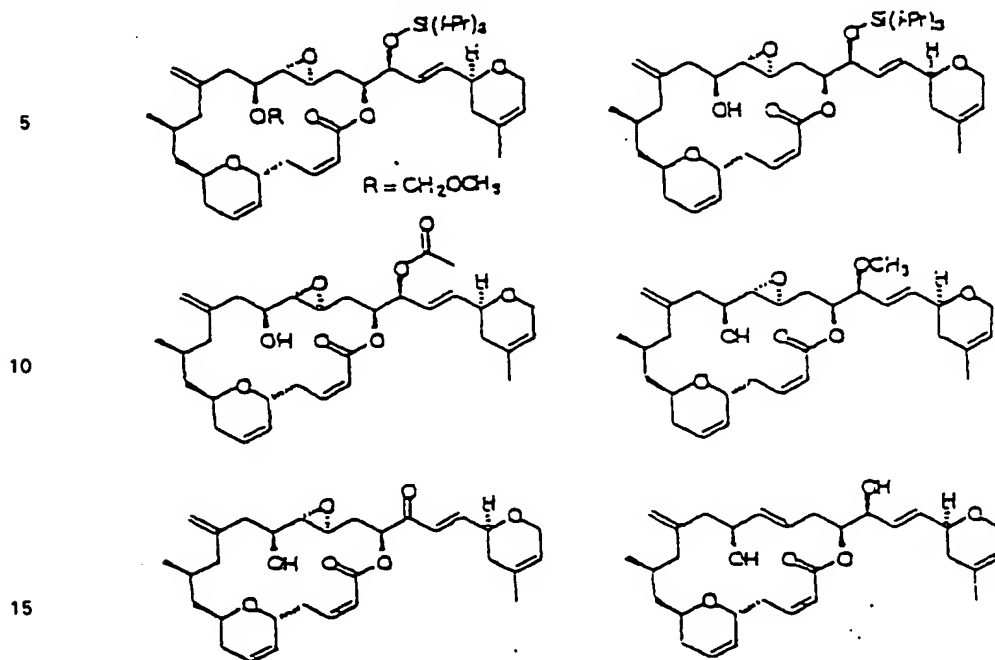
and variants known to exist in the basic laulimalide structure, including isolaulimalide. Such variants differ structurally from laulimalide as follows:



Isolaulimalide

Furthermore, synthetic variants of native laulimalide, termed analogs, which differ structurally from laulimalide as follows, are expected to retain the ability to inhibit the proliferation of hyperproliferative mammalian cells having a multiple drug resistant phenotype. Together with analog variants of laulimalide, the following representative examples are contemplated for use in the practice of the present invention (wherein A and B represent regions

where structural variation is permissible while retaining some measure of the activity of the title compound laulimalide):



A further aspect of the present invention includes compositions comprising the novel Laulimalide analog compounds.

20 Certain of the laulimalide compounds contemplated for use in the practice of the present invention were previously isolated from *Cacospongia mycofijiensis*, a marine sponge. These include Laulimalide and isolaulimalide. Generally, the native compounds are isolated as follows:

25 Lyophilized *C. mycofijiensis* was ground to a powder and extracted by stirring with dichloromethane/isopropanol for 24 hours at room temperature. The extraction was repeated under identical conditions with fresh solvent, and the combined extracts were evaporated in vacuo below 400°C. The residue was dissolved in 90% (v/v) aqueous methanol and extracted three times with
30 equal volumes of hexanes, and the hexane fractions were discarded. The aqueous methanol phase was diluted with water to 80% (v/v) aqueous

- 9 -

methanol and extracted three times with equal volumes of toluene. The combined toluene fractions were evaporated in vacuo below 400°C to dryness. The residue was dissolved in a minimum amount of dichloromethane and applied to a silica gel column equilibrated in ether, and the column was
5 eluted with ether. Active fractions were combined and rechromatographed over silica gel using a solvent system of 75% methyl-tert-butyl ether/25% hexanes (v/v) containing 1% isopropanol.

Although somewhat laborious, the structure of the laulimalides as a
10 class is amenable to chemical synthesis and the production of analogs, by techniques known in the art, is within the routine skill of an organic chemist. Thus, laulimalide analogs can be routinely synthesized while preserving some measure of the activity of the representative members of the class of compounds, especially their ability to inhibit the proliferation of a
15 hyperproliferative mammalian cell having a multiple drug resistant phenotype. Thus, novel laulimalide analogs having the structures set forth above are also considered to be within the scope of the present invention.

The novel and the previously disclosed laulimalide compounds of the
20 present invention can be therapeutically employed as anti-neoplastic agents and thereby used in methods to treat neoplastic diseases. As used herein, "neoplastic" pertains to a neoplasm, which is an abnormal growth, such growth occurring because of a proliferation of cells not subject to the usual limitations of growth. As used herein, "anti-neoplastic agent" is any
25 compound, composition, admixture, co-mixture or blend which inhibits, eliminates, retards or reverses the neoplastic phenotype of a cell.

Chemotherapy, surgery, radiation therapy, therapy with biologic response modifiers, and immunotherapy are currently used in the treatment of
30 cancer. Each mode of therapy has specific indications which are known to those of ordinary skill in the art, and one or all may be employed in an

- 10 -

attempt to achieve total destruction of neoplastic cells. Chemotherapy utilizing one or more laulimalides is provided by the present invention. Moreover, combination chemotherapy, chemotherapy utilizing laulimalides in combination with other neoplastic agents, is also provided by the subject
5 invention as combination therapy is generally more effective than the use of single anti-neoplastic agents. Thus, a further aspect of the present invention provides compositions containing a therapeutically effective amount of at least one new laulimalide compound of the present invention, including nontoxic addition salts thereof, which serve to provide the above-recited
10 therapeutic benefits. Such compositions can also be provided together with physiologically tolerable liquid, gel or solid carriers, diluents, adjuvants and excipients. Such carriers, diluents, adjuvants and excipients may be found in the United States Pharmacopeia Vol. XXII and National Formulary Vol XVII, U.S. Pharmacopeia Convention, Inc., Rockville, MD (1989), the contents of
15 which are herein incorporated by reference. Additional modes of treatment are provided in AHFS Drug Information, 1993 ed. by the American Hospital Formulary Service, pp. 522-660, the relevant contents of which are herein incorporated by this reference.

20 Certain embodiments of the present invention further provide that the pharmaceutical composition used to treat neoplastic disease contains at least one laulimalide compound and at least one additional anti-neoplastic agent. In further embodiments of the invention, the mammalian cell is human. Anti-neoplastic compounds which may be utilized in combination with laulimalides
25 include those provided in The Merck Index, 11th ed. Merck & Co., Inc. (1989) pp. Ther 16-17, the contents of which are hereby incorporated by reference. In a further embodiment of the invention, anti-neoplastic agents may be anti-metabolites which may include, for example, methotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, hydroxyurea, 2-chlorodeoxy-adenosine,
30 and the like. In another embodiment of the present invention, the anti-neoplastic agents contemplated are alkylating agents which may include, for

- 11 -

example, cyclophosphamide, melphalan, busulfan, paraplantin, chlorambucil, nitrogen mustard, and the like. In a further embodiment of the subject invention, the anti-neoplastic agents are plant-derived natural products which may include, for example, vincristine, vinblastine, paclitaxel, etoposide, and
5 the like. In a further embodiment of the present invention, the anti-neoplastic agents contemplated are antibiotics which may include, for example, doxorubicin (adriamycin), daunorubicin, mitomycin c, bleomycin, and the like. In a further embodiment of the subject invention, the anti-neoplastic agents contemplated are hormones which may include, for example, calusterone,
10 diomostavolone, propionate, epitiostanol, mepitiothane, testolactone, tamoxifen, polyestradiol phosphate, megestrol acetate, flutamide, nilutamide, trilotane, and the like. In a further embodiment of the subject invention, the anti-neoplastic agents contemplated include enzymes which may include, for example, L-Asparaginase or aminoacridine derivatives (e.g., amsacrine), and
15 the like. Additional anti-neoplastic agents include those provided in Skeel, Roland T., "Antineoplastic Drugs and Biologic Response Modifier: Classification, Use and Toxicity of Clinically Useful Agents," Handbook of Cancer Chemotherapy (3rd ed.), Little Brown & Co. (1991), the relevant contents of which are herein incorporated by this reference.

20

The present laulimalide compounds and compositions can be administered to mammals for veterinary use, such as for domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will vary according to the
25 type of use and mode of administration, as well as the particularized requirements of individual hosts. Ordinarily, dosages will range from about 0.001 to 1000 mg/kg, more usually 0.01 to 10 mg/kg, of the host body weight. Alternatively, dosages within these ranges can be administered by constant infusion over an extended period of time, usually exceeding 24
30 hours, until the desired therapeutic benefits have been obtained. Indeed, drug dosage, as well as route of administration, must be selected on the basis of

- 12 -

relative effectiveness, relative toxicity, growth characteristics of tumor and effect of laulimalides on cell cycle, drug pharmacokinetics, age, sex, physical condition of the patient, and prior treatment.

5 The laulimalide compounds, with or without additional anti-neoplastic agents, may be formulated into therapeutic compositions as natural or salt forms. Pharmaceutically acceptable non-toxic salts include the base addition salts (formed with free carboxyl or other anionic groups) which may be derived from inorganic bases such as, for example, sodium, potassium,
10 ammonium, calcium, ferric hydroxides, and the like; such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like. Such salts may also be formed as acid addition salts with any free cationic groups and will generally be formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic,
15 oxalic, tartaric, mandelic, and the like. Additional excipients which the further invention provides are those available to one of ordinary skill in the art, for example, that found in the United States Pharmacopeia Vol. XXII and National Formulary Vol XVII, U.S. Pharmacopeia Convention, Inc., Rockville, MD (1989), the relevant contents of which is herein incorporated by this
20 reference.

 The suitability of particular carriers for inclusion in a given therapeutic composition depends on the preferred route of administration. For example, anti-neoplastic compositions may be formulated for oral administration. Such
25 compositions are typically prepared either as liquid solution or suspensions, or in solid forms. Oral formulations usually include such normally employed additives such as binders, fillers, carriers, preservatives, stabilizing agents, emulsifiers, buffers and excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose,
30 magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations,

- 13 -

or powders, and typically contain 1%-95% of active ingredient, preferably 2%-70%.

Compositions of the present invention may also be prepared as

5 injectable, either as liquid solutions, suspensions, or emulsions; solid forms suitable for solution in, or suspension in, liquid prior to injection may be prepared. Such injectables may be administered subcutaneously, intravenously, intraperitoneally, intramuscularly, intrathecally, or intrapleurally. The active ingredient or ingredients are often mixed with diluents or

10 excipients which are physiologically tolerable and compatible with the active ingredient(s). Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH buffering agents.

15

The invention further provides methods for using laulimalide compounds encompassed by the genus structure to inhibit the proliferation of mammalian cells by contacting these cells with a laulimalide compound in an amount sufficient to inhibit the proliferation of the mammalian cell. A

20 preferred embodiment is a method to inhibit the proliferation of hyperproliferative mammalian cells. For purposes of this invention, "hyperproliferative mammalian cells" are mammalian cells which are not subject to the characteristic limitations of growth, e.g., loss of growth control and insensitivity to normal programmed cell death (apoptosis). A further

25 preferred embodiment is when the mammalian cell is human. The invention further provides contacting the mammalian cell with at least one laulimalide compound and at least one additional anti-neoplastic agent. The types of anti-neoplastic agents contemplated are the same as those disclosed hereinabove.

30

- 14 -

The invention further provides methods for using laulimalide compounds encompassed by the genus structure to inhibit the proliferation of hyperproliferative cells with drug-resistant phenotypes, including those with multiple drug-resistant phenotypes, by contacting said cell with a laulimalide compound in an amount sufficient to inhibit the proliferation of a hyperproliferative mammalian cell. A preferred embodiment is when the mammalian cell is human. The invention further provides contacting the mammalian cell with a laulimalide compound and at least one additional anti-neoplastic agent. The types of anti-neoplastic agents contemplated are the same as those disclosed hereinabove.

The present invention also provides methods of alleviating a pathological condition caused by hyperproliferating, multiple drug resistant mammalian cells. Invention methods comprise administering to a subject an effective amount of the pharmaceutical composition disclosed herein to inhibit proliferation of the cells. In further embodiments of the present invention, the mammalian cells are human.

In certain embodiments of the present invention, the method further comprises administering to the subject at least one additional therapy directed to alleviating the pathological condition. In certain embodiments of the present invention, the pathological condition is characterized by the formation of neoplasms. In further embodiments of the present invention, the neoplasms are selected from the group consisting of mammary, small-cell lung, non-small-cell lung, colorectal, leukemia, melanoma, pancreatic adenocarcinoma, central nervous system (CNS), ovarian, prostate, sarcoma of soft tissue or bone, head and neck, gastric which includes pancreatic and esophageal, stomach, myeloma, bladder, renal, neuroendocrine (which includes thyroid and non-Hodgkin's disease and Hodgkin's disease neoplasms), and the like.

- 15 -

The invention further provides a method for alleviating pathological conditions caused by hyperproliferating mammalian cells, for example, neoplasia, by administering to a subject an effective amount of a pharmaceutical composition provided hereinabove to inhibit the proliferation
5 of the hyperproliferating cells. As used herein "pathological condition" refers to any pathology arising from the proliferation of mammalian cells that are not subject to the normal limitations of cell growth. Such proliferation of cells may be due to neoplasms, including, for example, the following neoplasms: mammary, small-cell lung, non-small-cell lung, colorectal, leukemia,
10 melanoma, central nervous system (CNS), ovarian, prostate, sarcoma of soft tissue or bone, head and neck, gastric which includes pancreatic and esophageal, stomach, myeloma, bladder, renal, neuroendocrine (which includes thyroid and lymphoma, non-Hodgkin's and Hodgkin's), and the like. In a further embodiment of the invention, the neoplastic cells are human. The
15 present invention further provides methods of alleviating such pathological conditions utilizing laulimalide in combination with other therapies, as well as other anti-neoplastic agents. Such therapies and their appropriateness for different neoplasia may be found in Cancer Principles and Practice of Oncology, 4th ed., Editors DeVita, V., Hellman, S., and Rosenberg, S.,
20 Lippincott Co. (1993), the contents of which are herein incorporated by reference.

The present disclosure demonstrates that laulimalide compounds potently stabilize the microtubule structure in cultured cells. In addition, and
25 in contrast with the Vinca alkaloids and paclitaxel, laulimalide compounds are shown to be poor substrates for the drug-efflux pump P-glycoprotein.

The following examples serve to illustrate certain embodiments and aspects of the present invention and are not to be construed as limiting the
30 scope thereof. Thus, the following examples demonstrate the practice of the present invention in isolating, purifying and employing laulimalide microtubule

- 16 -

stabilizing agents for inhibiting the proliferation of a hyperproliferative mammalian cell having a multiple drug resistant phenotype.

Examples

5

In the examples which follow, all weights are given in grams (g), milligrams (mg), micrograms (μ g), nanograms (ng), or picograms (pg), all amounts are given in moles (mol), millimoles (mmol), micromoles (μ mol), nanomoles (nmol), picomoles (pmol), or femtomoles (fmol), all concentrations are given as percent by
10 volume (%), proportion by volume (v:v), molar (M), millimolar (mM), micromolar (μ M), nanomolar (nM), picomolar (pM), femtomolar (fM), or normal (N), all volumes are given in liters (L), milliliters (mL), or microliters (μ L), and linear measurements are given in millimeters (mm), or nanometers (nm) unless otherwise indicated.

15

Reagents

4,6-Diamidino-2-phenylindole (DAPI), sulforhodamine B (SRB), antibodies against β -tubulin, and Basal Medium Eagle containing Earle's salts (BME) were obtained from the Sigma Chemical Company (St. Louis, MO). Richter's medium
20 was obtained from BioWhittaker (Walkersville, MD) and Fetal Bovine Serum (FBS) was obtained from Hyclone Laboratories (Logan, UT).

Cell Lines

The A-10 rat aortic smooth muscle and SK-OV-3 human ovarian
25 carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in BME containing 10% FBS and 50 μ g/mL gentamycin sulfate. A sub-line of SK-OV-3 selected for resistance to vinblastine (SKVLB-1) was provided by Dr. Victor Ling (British Columbia Cancer Center, Vancouver, British Columbia) and was maintained in BME
30 containing 10% FBS and 50 μ g/mL gentamycin sulfate. The MDA-MB-435 human mammary adenocarcinoma cell line was obtained from Dr. Mai Higazi (Georgetown University, Washington, DC), and was maintained in Richters

- 17 -

medium containing 10% FBS and 50µg/mL gentamycin sulfate. Vinblastine was added to a final concentration of 1µg/mL to SKVLB-1 cells 24 hours after passage to maintain selection pressure for P-glycoprotein-overexpressing cells.

5

Inhibition of Cell Proliferation

The IC₅₀ for inhibition of cell proliferation was determined by measuring cell-associated protein after drug treatment using the sulforhodamine B assay.

10

Immunofluorescence Assays

A-10 cells were grown to 70-85% confluence on glass coverslips in BME supplemented with 10% FBS. Drug compounds in PBS were added to the indicated final concentrations and cells were incubated for an additional 24 hours.

15

For the staining of microtubules and intermediate filaments, the cells were fixed with cold methanol for 5 minutes, blocked for 20 minutes with PBS containing 10% calf serum to block nonspecific binding sites, and incubated at 37 °C for 90 min with monoclonal anti-β-tubulin at the dilutions recommended by the manufacturer. Bound primary antibodies were subsequently visualized by a one hour incubation with fluorescein(FITC)-conjugated sheep antimouse IgG (F-3008; Sigma). The coverslips were washed, stained with 0.1µg/mL DAPI for 10 minutes, mounted on microscope slides and the fluorescence patterns were examined and photographed using a Zeiss Axioplan microscope equipped with epifluorescence optics for fluorescein and DAPI.

20

25

Example 1

30 Effects of Laulimalide, Isolaulimalide, and Paclitaxel on Cellular Microtubules

- 18 -

Strong paclitaxel-like microtubule-stabilizing activity was found in the crude lipophilic extract from the marine sponge *C. mycofijiensis*. The extract was cytotoxic, and after treatment the only cell remnants that remained were thick, short microtubule bundles. Bioassay-directed purification of the extract yielded the microtubule active-compounds laulimalide and isolaulimalide, as well as the microfilament disrupter latrunuculin A. Studies with the purified compounds show that both laulimalide and isolaulimalide caused dramatic reorganization of cellular microtubules.

A-10 cells were treated with laulimalide, isolaulimalide, or paclitaxel for 18 hours and the morphological effects on microtubules examined by indirect immunofluorescence techniques. The control cells exhibited normal microtubules arrays with filamentous microtubules radiating from the microtubule organizing center to the cell periphery. Treatment of the cells with laulimalide disrupted the normal microtubule array; the microtubules were more numerous and appeared to occupy more of the cytoplasm. At 2 μ M, laulimalide exhibited bundles of short tufts of microtubules that were prevalent in the cell periphery and seemed to be independent of nucleation from microtubule organizing centers. Isolaulimalide at concentrations between 2-20 μ M caused an increase in the density of cellular microtubules, but no microtubule bundles were present.

Paclitaxel at concentrations between 1-20 μ M initiated the formation of a highly organized array of microtubules, some of which formed thick microtubule bundles. Long thick microtubule bundles often surrounded the nucleus. The extensive long microtubule hoops and bundles that formed after treatment with 2 μ M paclitaxel were not seen with laulimalide. Cellular microtubules stabilized with paclitaxel, laulimalide or isolaulimalide were resistant to vinblastine-induced depolymerization.

30

Example 2

Effects of Laulimalide and Isolaulimalide on Nuclear Structure

A characteristic of both A-10 and SK-OV-3 cells treated with a wide
5 range of concentrations of laulimalide and isolaulimalide was the formation of multiple micronuclei. The effects of laulimalide on nuclear structure are visible: The normal rounded shape of the nucleus, which is devoid of microtubules, can be detected, whereas in laulimalide-treated cells this distinct microtubule-free area containing the discrete central nucleus was lost
10 and only vesicle-like areas devoid of microtubules remained. Nuclear staining of control cells revealed a central compact nucleus, whereas laulimalide-treated cells exhibited a dramatic breakdown of the nucleus into micronuclei. Similar nuclear changes occurred with isolaulimalide. Paclitaxel also initiated the formation of micronuclei, as has been reported by others. Analysis of the
15 incidence of micronuclei formation showed that a higher percentage of laulimalide-treated cells contained micronuclei than cells treated with the same concentrations of paclitaxel.

The data in Figure 1 demonstrate that 0.02-2 μ M laulimalide caused
20 micronuclei formation in approximately 60% of A-10 cells. Paclitaxel at the same concentrations caused approximately 40% of the cells to exhibit this abnormal restructuring of the nucleus. At all concentrations tested, laulimalide caused a higher percentage of cells to exhibit micronuclei when compared with paclitaxel-treated cells. The difference between the two drugs
25 was statistically significant at all five concentrations tested ($P < 0.0001$).

Example 3

Effects of Laulimalide on Cell Cycle Progression and Mitotic Spindles

30 A common characteristic of anti-microtubule agents is their ability to initiate mitotic arrest. Flow cytometric analysis revealed that laulimalide-

- 20 -

induced cell cycle arrest in G2-M in MDMB-435 breast carcinoma cells within nine hours of treatment (Figure 2). This is consistent with the effects of other antimicrotubule agents, where disruption of microtubule dynamics prevents normal mitotic progression and leads to mitotic arrest. Abnormal
5 mitotic spindles were seen in both A-10 cells and SK-OV-3 cells after treatment with laulimalide and isolaulimalide. The mitotic cells were rounded, and the spindles formed a circular pattern with spindle microtubules radiating outward from a microtubule-free core in the center of the cell. In the mitotic cells, the nuclear membranes were not apparent, and the chromatin was
10 condensed and aligned in a circular pattern. Abnormal mitotic spindles were visible in paclitaxel-treated cells and were typically tri-or tetra-polar and did not exhibit the morphology seen with the laulimalides.

Example 4

15 Effects of Laulimalide, Isolaulimalide, and Paclitaxel on Cell Proliferation of Drug-Sensitive and Multidrug-Resistant Cell Lines

The early literature on laulimalide reported that it was a cytotoxin, but without specifying a mechanism. Experiments were conducted to determine
20 the IC₅₀ values for laulimalide and isolaulimalide in two drug-sensitive cell lines, MDA-MB-435 and SK-OV-3, and in a multidrug-resistant cell line, SKVLB-1. Cells were treated with varying concentrations of the drugs for 48 hours, and cell-associated protein was determined using the SRB assay. The IC₅₀ for each compound was calculated for each cell line.

25

Laulimalide was found to be a potent inhibitor of cell proliferation with IC₅₀ values between 5-12 nM (Table 1).

- 21 -

Table 1

<u>Cell Line</u>	<u>Laulimalide</u>	<u>Isolaulimalide</u>	<u>Paclitaxel</u>
MDA-MB-435	5.74 \pm 0.58	1970 \pm 97	1.02 \pm 0.25
SK-OV-3	11.53 \pm 0.53	2570 \pm 290	1.71 \pm 1.07
5 SKVLB-1	1210 \pm 490	2650 \pm 1384	> 100,000
Resistance Factor	105	1.03	> 58,480

The values presented in Table 1 represent the means of three (laulimalide, isolaulimalide) or four (paclitaxel) experiments \pm SD.

10

Isolaulimalide is less potent with IC₅₀ values in the low μ M range. Both laulimalide and isolaulimalide inhibited the proliferation of the SKVLB-1 cell line that overexpresses the drug efflux pump P-glycoprotein. The resistance factors between the parental, drug-sensitive line (SK-OV-3) and the drug-resistant cell line (SKVLB-1) were 105 and 1.03 for laulimalide and isolaulimalide, respectively (Table 1). Greater than 70% inhibition of the SKVLB-1 cell line was not achieved with concentrations of paclitaxel up to 100 μ M. Laulimalide and isolaulimalide are significantly more effective against the SKVLB-1 cell line than paclitaxel. These data confirm that these new agents are poor substrates for transport by P-glycoprotein.

20

The IC₅₀ for inhibition of proliferation was also determined in the A-10 cell line, a nontransformed line that was used to show the effects of microtubule-stabilizing agents on cellular structures. The IC₅₀ for laulimalide was 51 μ M and for paclitaxel was 40 μ M. The nontransformed A-10 cell line was much less sensitive to the inhibitory effects of both paclitaxel and laulimalide, illustrating the need to use micromolar concentrations in these cells to induce abnormal microtubules.

25

Example 5**Initiation of Apoptosis by Laulimalide**

The ultimate mechanism of action of many cytotoxic cancer
5 chemotherapeutic agents is the initiation of pathways of gene and protein
expression leading to apoptosis. Antimicrotubule drugs including paclitaxel,
vinblastine, and cryptophycin 1 initiate apoptosis both in vitro and in vivo.
The flow cytometry data (Figure 2) show a doubling of the subdiploid peak at
18 hours (Figure 2C), suggesting the initiation of apoptosis. The loss of
10 cellular DNA is detected by the appearance of the subdiploid peak when
apoptotic cells are analyzed by flow cytometry. Studies were undertaken to
determine whether laulimalide initiates a gene-driven program of cellular
suicide.

15 During apoptosis, specific cysteine proteases called the caspases are
activated. Activation of the caspase cascade leads to the proteolytic
degradation of specific cellular proteins. The activation of caspase 3 and the
proteolysis of the DNA repair enzyme PARP, a downstream substrate of
caspase 3, were examined in cell lysates from laulimalide-treated cells.
20 Activation of caspase 3 leads to the loss of the 32kDa proenzyme and the
formation of the activation products p17 and p12. Analysis of immunoblot
data from cell lysates shows the formation of the p17 activation product at
24, 42 and 48 hours after laulimalide treatment. The loss of the p32
proenzyme is seen at 48 hours. The specific proteolysis of PARP by caspase
25 3 leads to the formation of two products, an 89kDa COOH-terminal fragment
and a 24kDa N-terminal fragment. The proteolysis of PARP and the
appearance of the 89kDa degradation product coincided with the activation of
caspase 3. Caspase 3 was activated and PARP proteolytically cleaved in cell
lysates from cells treated with laulimalide for 42 and 48 hours. These data
30 are consistent with laulimalide-induced apoptotic cell death.

Example 6**Effects of Laulimalide on Tubulin Polymerization in Vitro**

One characteristic of the microtubule-stabilizing agents paclitaxel, discoldermolide, epothilones A and B, and eleutherobin is the ability of these agents to initiate the polymerization of tubulin in the absence of polymerization promoters, such as glycerol. Studies were undertaken to determine the effects of laulimalide on tubulin polymerization. Both isolaulimalide and laulimalide stimulated tubulin polymerization. Figure 3A shows the effects of laulimalide on tubulin polymerization, and the accompanying Figure 3B shows the effects of paclitaxel. Comparisons between the effects of laulimalide and paclitaxel on tubulin polymerization (Figure 3, A and B) show that, at low micromolar concentrations, paclitaxel is more potent than laulimalide (see also Table 2).

Table 2

<u>Drug</u>	<u>EC₅₀</u>
Laulimalide	4.32 ± 0.4
Paclitaxel	1.44 ± 0.06

At low micromolar concentrations more tubulin polymer was formed in the presence of paclitaxel, and the rate of polymerization was faster than was seen with equivalent concentrations of laulimalide.

25

A very different relationship was seen when comparing the 20μM concentrations of laulimalide and paclitaxel. The 20μM concentration of laulimalide was more effective at stimulating the formation of tubulin polymer than was the 20μM concentration of paclitaxel (Figures 3A and 3B). Laulimalide promoted the polymerization of approximately 30% more tubulin polymer than was formed in the presence of paclitaxel, and the kinetics of

- 24 -

tubulin formation were twice as fast as the rate measured in the presence of 20 μ M paclitaxel.

The tubulin polymers that formed in the presence of laulimalide were
5 insensitive to cold and CaCl₂-induced depolymerization. Neither paclitaxel nor laulimalide promoted the polymerization of tubulin at 0°C, and laulimalide stabilized tubulin polymer formed in the presence of GTP.

Samples of the tubulin polymer formed were examined by electron
10 microscopy to determine whether the increase in turbidity measured during the polymerization experiments was due to the formation of microtubule-like polymers or the formation of other structures. Under high magnification (x63,000) the tubulin polymers formed by paclitaxel and laulimalide were indistinguishable. Both agents formed structures resembling tubules with
15 evidence of longitudinal symmetry. Examination of tubulin polymers formed in the presence of the microtubule-stabilizing agents at lower magnification (x4,000) showed that the polymers formed with laulimalide were very long structures with rounded curves. The polymers formed with paclitaxel were notably shorter and exhibited no rounded curves, but instead formed linear
20 structures with angular branches.

Laulimalide and isolaulimalide were first isolated on the basis of their cytotoxicity, however, the mechanism of action was not elucidated. These agents have now been shown to be paclitaxel-like stabilizers of microtubules
25 that cause alterations of both interphase and mitotic microtubules. Laulimalide is a potent inhibitor of cell proliferation and initiates mitotic arrest, micronuclei formation, and ultimately apoptosis. These compounds are superior to paclitaxel in their ability to circumvent P-glycoprotein-mediated drug resistance. The laulimalides represent a new class of paclitaxel-like
30 microtubule-stabilizing agents with properties that may provide advantages over the taxanes.

- 25 -

Laulimalide and isolaulimalide are chemically related compounds, with isolaulimalide being a decomposition product of laulimalide. The difference between these two compounds is in the size and attachment points of the oxygen-containing ring within the top portion of the molecules. Laulimalide contains a three-membered epoxide ring involving carbons C-16 and C-17, whereas isolaulimalide contains a five-membered tetrahydrofuran ring linking carbon C-17 with side chain carbon C-20. This slight chemical difference between laulimalide and isolaulimalide results in a difference in potency of greater than two orders of magnitude in their ability to inhibit cell proliferation. Furthermore, this site also seems to be crucial for recognition by the multidrug efflux pump P-glycoprotein. Laulimalide had a resistance factor of 105 when comparing the IC₅₀ values in SK-OV-3 cells and SKVLB-1 cells, whereas isolaulimalide was equally sensitive in both cell lines. These data demonstrate that the epoxide moiety of laulimalide is critical for its interaction with both tubulin and P-glycoprotein.

Among the five groups of known antimicrotubule agents having paclitaxel-like microtubule-stabilizing properties, laulimalide most closely resembles the epothilones. Although the ring size of laulimalide is two carbons larger (18-membered versus 16-membered), both contain a similar structural motif that incorporates the epoxide ring, an unsaturated side chain bearing a methylated heterocyclic ring, and the ester of the macrocyclic lactone ring. This similarity seems to translate to similar activities. Both stabilize microtubules, and like laulimalide, the epothilones are poor substrates for P-glycoprotein-mediated transport.

The cellular effects of laulimalide are similar to, but distinct from, the cellular effects of paclitaxel. The increase in the density of cellular microtubules observed at low concentrations of laulimalide closely resembled the changes induced by paclitaxel at the same concentrations. However, at

- 26 -

concentrations above 2 μ M, the effects diverged and laulimalide initiated short thick bundles of microtubules that were more prevalent in the cell periphery and appeared to form from many nucleation centers. These effects are consistent with the greater efficacy of 20 μ M laulimalide in promoting the polymerization of purified tubulin. In contrast, paclitaxel-induced microtubule bundles were long and thick and aligned in the central areas of the cells surrounding the nucleus, consistent with nucleation from one or two centers. Long microtubule bundles were not seen in cells treated with a wide range of laulimalide concentrations. These data demonstrate that in cells, laulimalide was not as effective as paclitaxel at elongation of microtubules, but was more effective at stimulating the formation of microtubules from multiple nucleation centers, resulting in shorter microtubule bundles in the periphery rather than the long microtubule bundles surrounding the nucleus.

The microtubules that form the mitotic spindle are highly dynamic structures and are more sensitive to disruption by antimicrotubule agents than are the less dynamic interphase microtubules. Agents that target microtubules disrupt mitotic spindle dynamics, thereby preventing normal mitosis, leading to mitotic arrest. Mitotic spindles formed in the presence of laulimalide were abnormal and formed unique starburst arrays in contrast to the short thickened tri- and tetra-polar spindles formed in the presence of paclitaxel. Laulimalide-treated mitotic cells exhibited chromatin condensation, loss of the nuclear envelope and abnormal chromatin alignment. The aberrant mitotic spindles were associated with circular chromatin arrays, suggesting that the microtubules were coordinating a specific, but abnormal structuring of the chromatin. Disruption of the mitotic apparatus by laulimalide treatment lead to mitotic arrest, followed by the initiation of apoptosis, as determined by the increase in cells in G2-M and the activation of the caspase cascade.

- 27 -

Cells treated with laulimalide exhibited vesicle-like structures in the central region of the cell. DAPI staining revealed that these structures were composed of DNA and that laulimalide initiated the formation of multiple micronuclei. In A-10 cells, micronuclei were found in the majority of cells
5 with treatments of 20nM-2μM laulimalide. Micronuclei formations occur as a consequence of treatment with either paclitaxel or epothilones and are thought to be the result of abnormal mitosis leading to abnormal chromosome segregation.

10 One characteristic of the paclitaxel-like microtubule-stabilizing agents is their ability to promote the polymerization of tubulin. Normally tubulin will not polymerize without tubulin promoters; however, laulimalide, like paclitaxel, stimulated the polymerization of tubulin in the absence of microtubule-associated proteins and glycerol. The rate and extent of
15 polymerization in the presence of laulimalide was dependent on concentration and differed significantly from paclitaxel, suggesting that there are differences in their mechanisms of action. Further experimentation in the presence of difference tubulin and laulimalide concentrations and in the presence and absence of microtubule-associated proteins will allow more comprehensive
20 comparisons of the tubulin polymerizing effects of laulimalide as compared with paclitaxel, discodermolide, and the epothilones.

The tubulin polymers formed in the presence of laulimalide were indistinguishable from paclitaxel-induced polymers under high magnification,
25 but were noticeably longer than the paclitaxel-induced polymers when visualized under lower magnification. Under these conditions, laulimalide seems to promote polymer-elongating activity more readily than paclitaxel. These in vitro effects with purified tubulin differ from the effects in cells where paclitaxel promotes longer microtubules, whereas laulimalide promotes
30 short microtubules. The initial results demonstrate that there are intriguing differences in the mechanisms of action of laulimalide and paclitaxel.

- 28 -

The clinical success of the taxanes in treating a wide range of tumors has lead to the search for new agents with a similar mechanism of action. The recent discovery of the beneficial activities of the class of laulimalides
5 provides new drugs which are expected to prove useful to treat cancer.

All patents and patent applications cited in this specification are hereby incorporated by reference as if they had been specifically and individually indicated to be incorporated by reference.

10

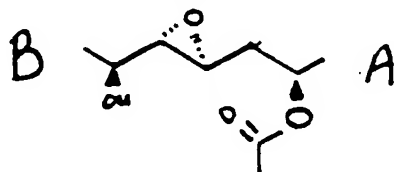
Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those of ordinary skill in the art in light of the disclosure that certain changes and modifications may be made thereto without
15 departing from the spirit or scope of the appended claims.

- 29 -

WHAT IS CLAIMED IS:

1. A method of inhibiting the proliferation of a hyperproliferative mammalian cell having a multiple drug resistant phenotype, said method comprising contacting the cell with an amount of a laulimalide compound
 5 effective to disrupt the dynamic state of microtubule polymerization and depolymerization to arrest cell mitosis, thereby inhibiting the proliferation of the cell.

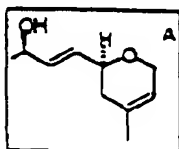
2. The method according to claim 1 wherein the laulimalide
 10 compound is a compound of Structure I as follows:



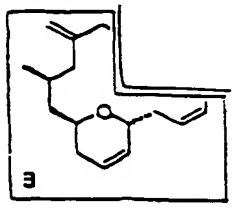
wherein:

15 the epoxide ring in the formula can optionally be replaced with a double bond, and

A is a structure in accordance with the formula:



20 B is a structure in accordance with the formula:

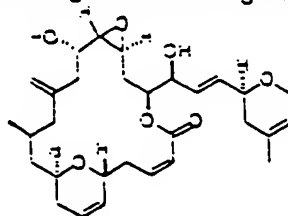


25 with the proviso that at least one of regions A or B includes a structural modification which distinguishes the structure from that depicted in Structure I while preserving the ability of the compound to disrupt the dynamic state of microtubule polymerization and depolymerization to arrest cell mitosis,
 30 thereby inhibiting the proliferation of the cell.

- 30 -

3. The method according to claim 1 wherein the laulimalide compound is laulimalide having the following structure:

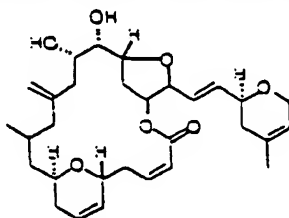
5



Laulimalide

4. The method according to claim 1 wherein the laulimalide compound is isolaulimalide having the following structure:

10

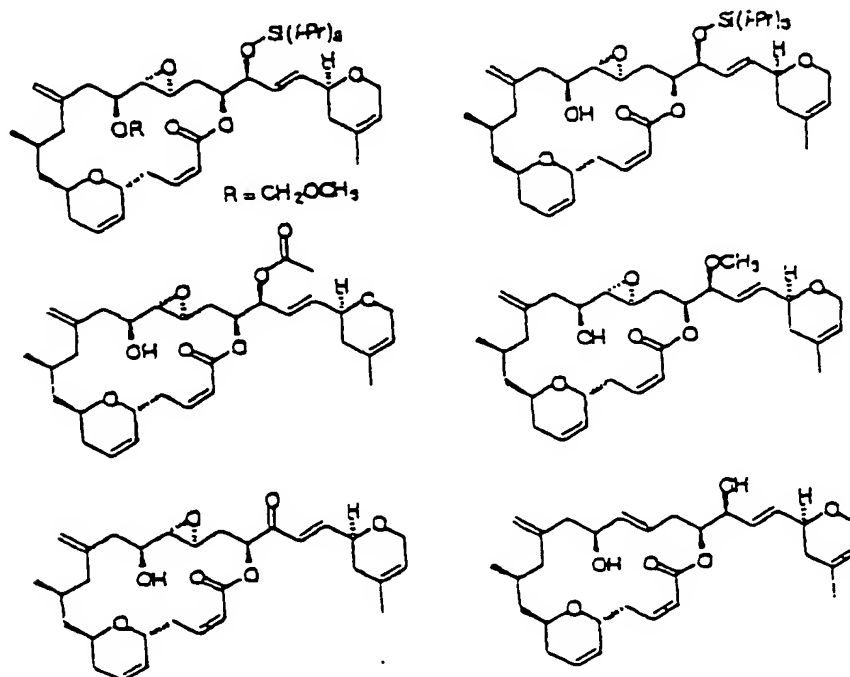


15

Isolaulimalide

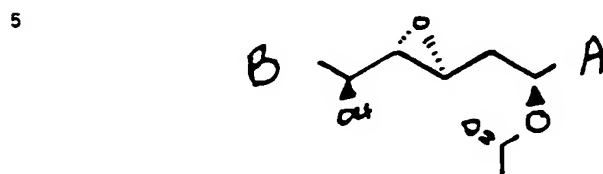
5. The method according to claim 1 wherein the laulimalide compound is selected from the group consisting of:

20



- 31 -

6. A therapeutic composition comprising at least one laulimalide compound according to the following formula

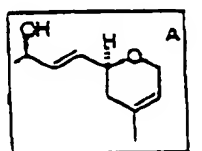


10

wherein,

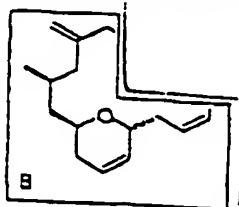
A is a structure in accordance with the formula:

15



B is a structure in accordance with the formula:

20

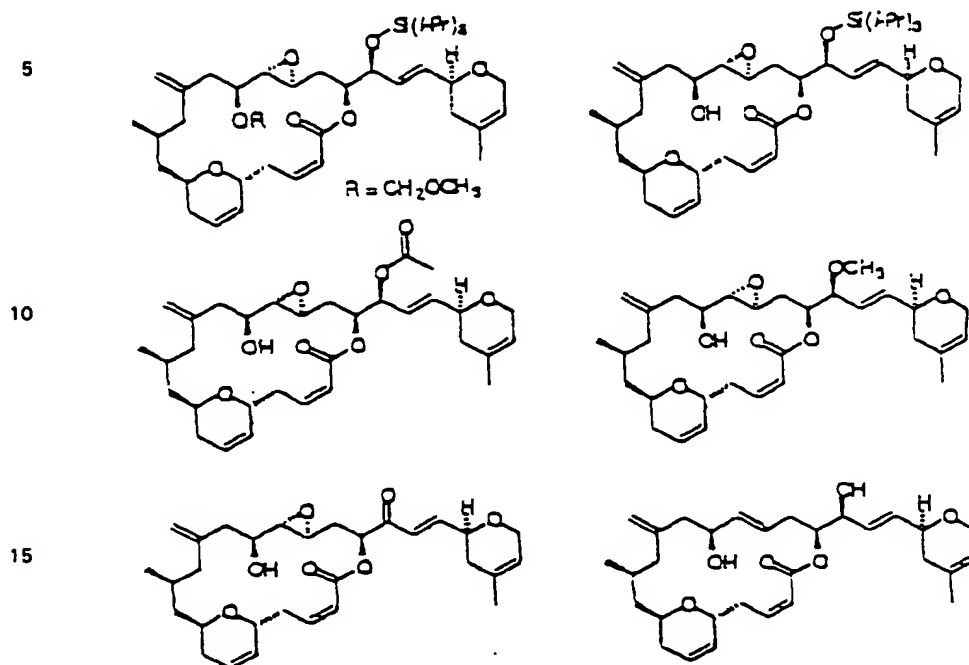


with the proviso that at least one of regions A or B includes a structural modification which distinguishes the structure from that depicted in Structure I, while preserving the ability of the compound to disrupt the dynamic state of microtubule polymerization and depolymerization to arrest cell mitosis, thereby inhibiting the proliferation of the cell.

25

- 32 -

7. The composition according to claim 6 wherein the laulimalide compound is a member selected from the group consisting of the following structures:



8. The composition according to claim 6 further comprising an anti-neoplastic agent.

20

9. A method of alleviating a pathological condition caused by hyperproliferating, multiple drug resistant mammalian cells, said method comprising administering to a subject an effective amount of the pharmaceutical composition according to claim 6 to inhibit proliferation of the

25 cells.

1/2

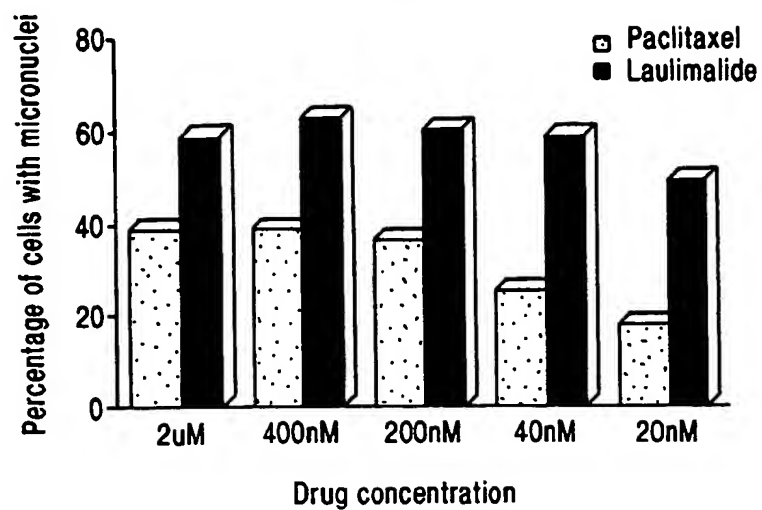


FIG. 1

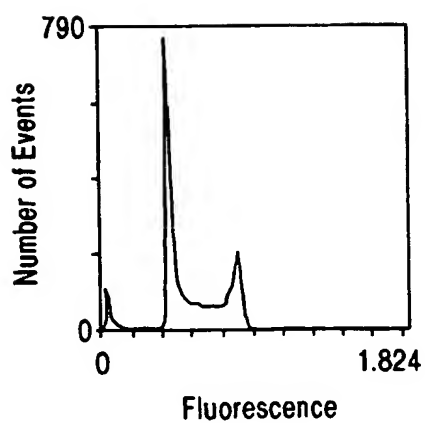


FIG. 2A

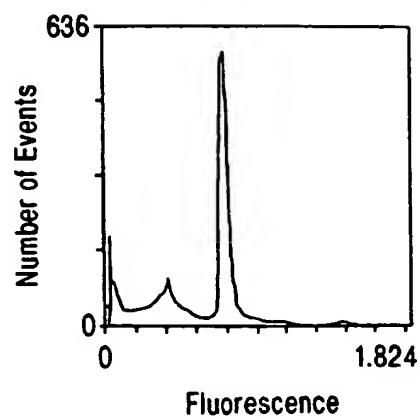


FIG. 2C

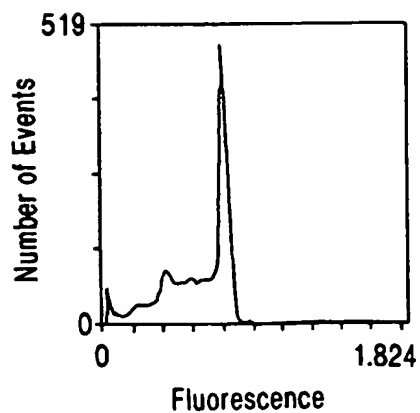


FIG. 2B

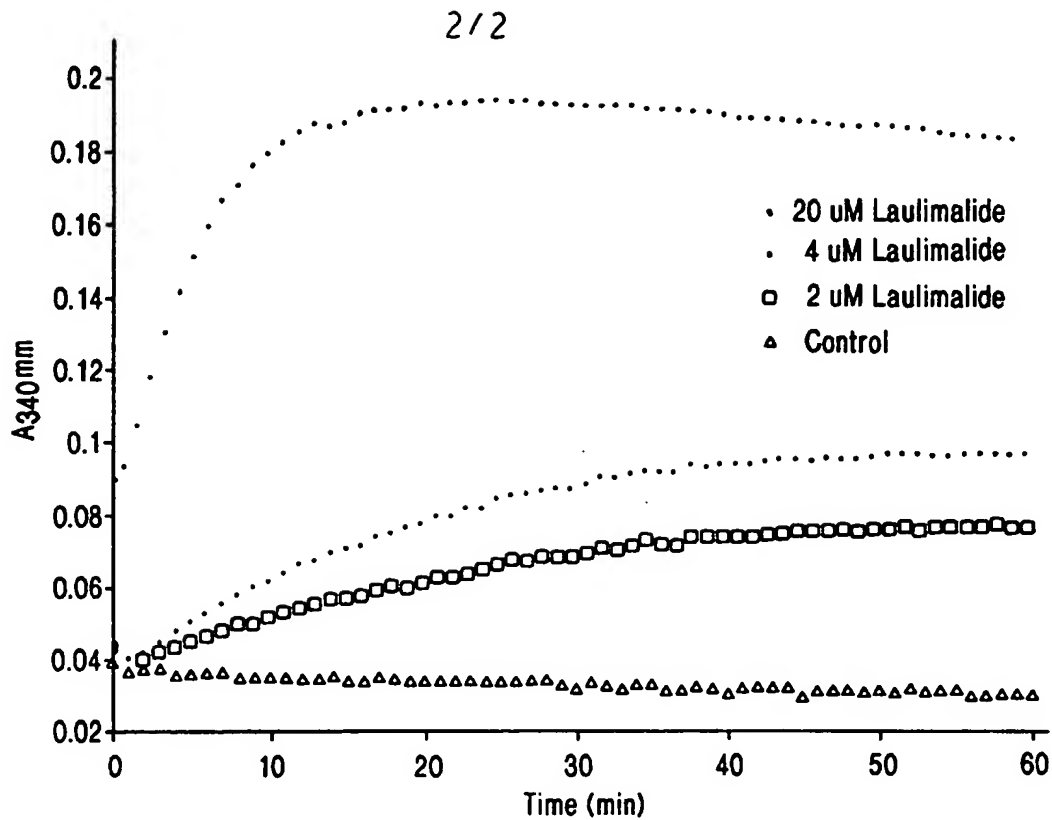


FIG. 3A

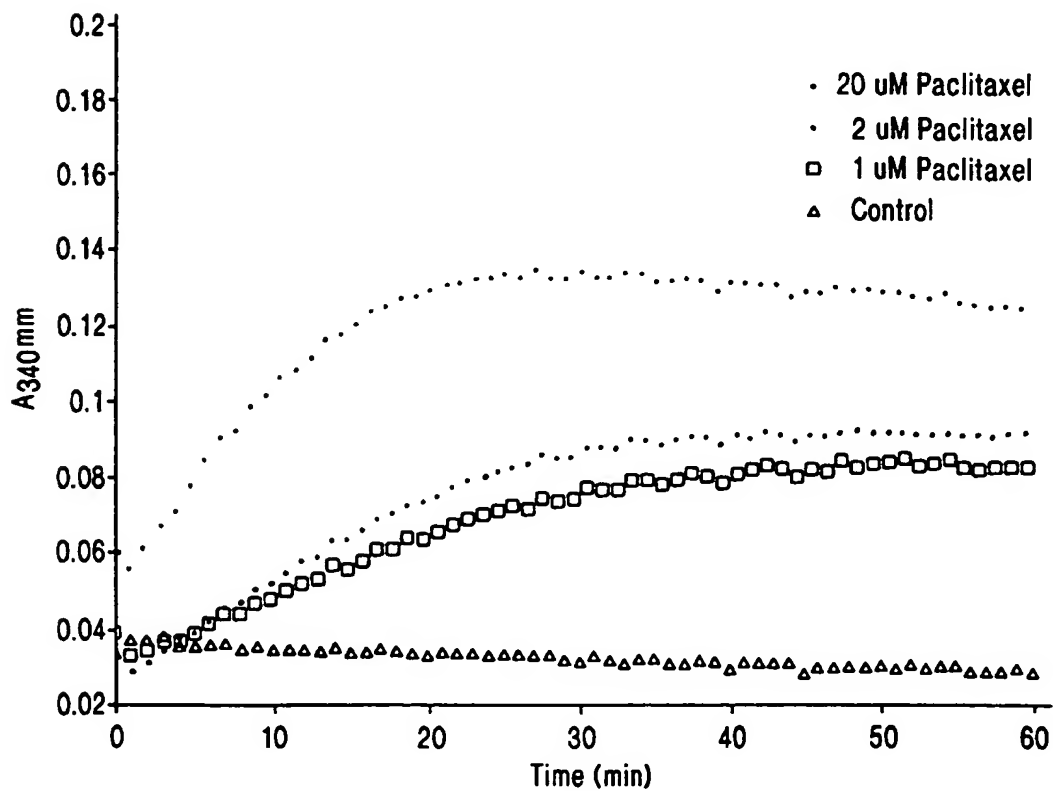


FIG. 3B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/02590

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/365 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; MOOBERRY, SUSAN L. ET AL: "Laulimalide and isolaulimalide, new paclitaxel-like microtubule- stabilizing agents" retrieved from STN Database accession no. 130:291200 CA XP002167919 abstract & CANCER RES. (1999), 59(3), 653-660 , 1999, XP000999521 --- -/--	1-9



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed.

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

21 May 2001

Date of mailing of the international search report

04/07/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Trifilieff-Riolo, S

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/02590

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; TANAKA, JUN-ICHI ET AL: "New cytotoxic macrolides from the sponge Fasciospongia rimosa" retrieved from STN Database accession no. 124:312574 CA XP002167920 abstract & CHEM. LETT. (1996), (4), 255-6 , 1996, XP000999521 page 255 -----	1-9
X	WO 97 10242 A (PHARMA MAR SA ;RUFFLES GRAHAM KEITH (GB); HIGA TATSUO (JP); TANAKA) 20 March 1997 (1997-03-20) claim 1 -----	1-4,6,9

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/02590

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9710242 A	20-03-1997	AU 6936596 A	01-04-1997